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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:	Examiner: J. Brusca
COX III et al.	Group Art Unit: 1631
For: **REGULATION OF ENDOGENOUS GENE EXPRESSION IN CELLS USING ZINC FINGER PROTEINS**	Confirmation No.: 6940
Serial No.: 09/706,243	**SECOND DECLARATION PURSUANT TO 37 C.F.R. § 1.132 OF CASEY C. CASE, PhD.**
Filed: November 3, 2000	
Atty. Docket No.: 8325-0002.10 (S2-US3)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

I, Casey C. Case, hereby declare as follows:

1. I signed a Declaration Pursuant to 37 C.F.R. § 1.132 regarding the above-referenced patent application on October 16, 2003 (hereinafter "the first Case declaration"). My qualifications and the documents I reviewed were set forth in the first Case declaration.

2. As detailed in the first Case declaration, I reiterate herein that various peptide sequences have been shown to act as membrane translocating, or internalization sequences, meaning that when they are added to the amino acid sequence of a protein that ordinarily does not pass through the plasma membrane, the resulting chimeric protein can now pass through the membrane and enter the cytoplasm. A peptide from the Tat transcription factor of HIV is one well-known example of a membrane translocating sequence. Another example is the peptide from the third helix (amino acids 43-58) of the *Drosophila* homeodomain transcription factor *Antennapedia* (Gehring et al. (1994) Annu. Rev. Biochem. 63: 487-526. This amphipathic peptide (N-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-C) has been shown to enter the cell in a receptor-independent manner (Derossi et al (1996) J. Biol. Chem. 271: 18188-18193). Tat and *Antennapedia* peptides and their use as membrane translocations peptides is discussed on page 44, lines 7-21 of the specification.

3. The data presented in the first Case declaration were prepared in collaboration with Dr. Frank Giordano and his colleagues at the Yale School of Medicine. This data demonstrated that the *Antennapedia* internalization peptide (as well as other internalization peptides selected from a phage display experiment) can be used to create chimeric Zinc Finger Transcription Factors (ZFP-TFs) that are able to cross the plasma membrane, enter the nucleus and activate their intended target gene. As detailed in the first Case declaration, this data was summarized in abstract form (Yeh et al. discussed in the first Case Declaration) and in an oral presentation at the 2003 meeting of the American Society for Gene Therapy. Further experimental details are described in Exhibit A, "Experimental Protocols," attached hereto.

4. Attached to the first Case Declaration was Exhibit B, which included Figures 1-6. To clarify, Figure 5 of the first Case Declaration shows results of increased VEGF expression *in vitro* after administration to cells of a fusion protein comprising a VEGF-targeted zinc finger protein and an *Antennapedia* (AP) membrane translocation peptide. *Antennapedia* peptides used in the fusion proteins are those as described on page 44, lines 10-13 and lines 20-21 of the specification as filed.

5. Thus, Figure 5 as attached to the first Case Declaration shows that protein-delivery mediated by *Antennapedia* effectively transports VEGF-targeted ZFPs into a cell and, furthermore, that the ZFPs are functional (*i.e.,* effectively upregulate endogenous VEGF expression) following *in vitro* administration in protein form.

6. Data, attached hereto, show that ZFPs prepared and delivered as peptides according to the teachings of the specification can be utilized *in vivo* to modulate gene expression and facilitate angiogenesis. In particular, an established model system for determining angiogenesis is evaluation of vessel formation in the murine ear. Using this system, the number of blood vessels observed after injection of a mouse ear with ZFP-*Antennapedia* fusion proteins was evaluated. Exhibit A, entitled "Experimental Protocols," provides details on these and other protocols used in our studies. As set forth in Exhibit A, administration of VEGF-targeted ZFPs to live animals was conducted essentially as described on page 49, lines 10-13 and 27.

7. Exhibit B attached hereto contains photographs showing increased vascularity in mouse ear following injection of ZFP in the ear. The left photograph shows an ear that had been injected with a protein comprising a VEGF-targeted ZFP (VOP32E), a transcriptional activation domain and the *Antennapedia* translocation domain. The right photograph shows an ear that had been injected with an internalization peptide only. Comparison of the two photographs indicates that the ZFP-injected ear show a higher degree of vascularity. Thus, using the accepted live mouse model system for angiogenesis, it has been demonstrated that VEGF-targeted ZFPs

delivered as proteins using an *Antennapedia* translocation domain as described in the specification, modulate vessel formation.

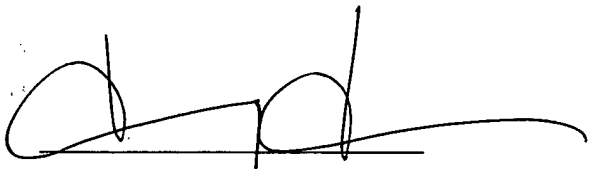
8. In addition, protein-mediated delivery of VEGF-targeted ZFPs enhanced VEGF expression in muscle *in vivo*. Exhibit C shows increased VEGF expression in mouse skeletal muscle obtained from a mouse injected in a hindlimb muscle with a solution containing a fusion protein comprising a VEGF-targeted ZFP, a transcriptional activation domain and the *Antennapedia* translocation domain. Internal standards indicated that enhancement of VEGF expression in these tissues is not due to injection of protein *per se*. These studies demonstrate that ZFPs, administered as fusion proteins comprising an *Antennapedia* translocation domain according to the teachings of the specification, regulate gene expression *in vivo*.

9. Therefore, I reiterate the conclusions set forth in Dr. Pabo's Declaration and in the first Case Declaration, namely that, as a technical matter, a skilled worker could have readily delivered ZFPs in protein form to cells in view of the teachings of the specification, together with the common general knowledge, tools and methods available as of the effective filing date of January 1999.

10. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3-11-04

Date



Casey C. Case, Ph.D.

Experimental Protocols

Protein delivery constructs were made by inserting a sequence encoding either *Antennapedia* (amino acids 43-58) (AP), or a specific internalization peptide obtained by phage display (IP), into operative linkage with sequences encoding VOP32E, a VEGF-A activating ZFP that has a nuclear translocation sequence in operative linkage with a VEGF-targeted ZFP-DNA binding domain fused to the p65 activation domain. The ability of the VOP32E ZFP-TF to activate VEGF transcription has been well established (Liu et al. (2001) J. Biol. Chem. 276: 11323-11334). The protein delivery constructs were subcloned into an expression vector that contains a multiple histidine tag, allowing purification of the fusion proteins. The resulting plasmids were then used to transform *E. coli* BL21 and transformed cells were grown to an optimum O.D. prior to harvesting of the proteins. The cells were sonicated, and the resulting crude lysates were incubated with Nickel-agarose beads under non-denaturing conditions. After multiple washes the proteins were eluted from the beads, collected and then dialyzed against phosphate buffered saline prior to use *in vitro* or *in vivo*.

The ability of the fusion protein to induce angiogenesis *in vivo* was tested using the established mouse ear angiogenesis model. Three days after injection of an *Antennapedia*-VOP32E fusion protein into a mouse ear, a marked increase in vascularity was observed, compared with the contralateral ear that had been injected with the internalization peptide alone (*i.e.*, not fused to a ZFP) (See Exhibit B).

To demonstrate the ability of these fusion peptides to activate gene expression in an intact living organism (*in vivo*), purified fusion proteins (either a fusion to the *Antennapedia* Internalization Peptide (AP) or one derived from phage display (IP), both described above) were injected into murine hindlimb muscles. As a control, the AP or IP peptide alone (not fused to a ZFP) was injected into the contralateral limb. Three days later the muscles were harvested, RNA was prepared and tested for VEGF-A mRNA levels using real-time PCR (Taqman®). Increases in VEGF mRNA levels in the fusion protein-injected muscles are expressed relative to the control muscle samples. (See Exhibit C)

VOP32E
Protein
Transduction

Control Protein
Transduction